

# **EXHIBIT A**

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# Selective Estrogen Receptor Modulator Idoxifene Inhibits Smooth Muscle Cell Proliferation, Enhances Reendothelialization, and Inhibits Neointimal Formation In Vivo After Vascular Injury

Tian-Li Yue, PhD; Lynne Vickery-Clark, BS; Calvert S. Loudon, PhD; Juan-Li Gu, MD; Xin L. Ma, MD, PhD; Padma K. Narayanan, PhD; Xiang Li, MS; Jun Chen, MS; Barbara Storer, BS; Robert Willette, PhD; Kent A. Gossett, PhD; Eliot H. Ohlstein, PhD

**Background**—Idoxifene (ID) is a tissue-selective estrogen receptor modulator (SERM). The pharmacological profile of ID in animal studies suggests that it behaves like an estrogen receptor (ER) agonist in bone and lipid metabolism while having negligible ER activity on the reproductive system. It is unknown whether ID retains the vascular protective effects of estrogen.

**Methods and Results**—In cultured vascular smooth muscle cells (VSMCs), ID inhibited platelet-derived growth factor–induced DNA synthesis and mitogenesis with  $IC_{50}$  values of 20.4 and 27.5 nmol/L, respectively. Treatment with ID resulted in S-phase cell cycle arrest in serum-stimulated VSMCs. ID 1 to 100 nmol/L significantly protected endothelial cells from tumor necrosis factor- $\alpha$  (TNF- $\alpha$ )–induced apoptosis in vitro. Virgin Sprague-Dawley rats ovariectomized 1 week before the study were treated with ID ( $1 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{d}^{-1}$ ) or vehicle by gavage for 3 days before balloon denudation in carotid artery. The SMC proliferation in injured vessels was determined by immunostaining for proliferating cell nuclear antigen (PCNA). The number of PCNA-positive SMCs was reduced by 69%, 82%, and 86% in the media at days 1, 3 and 7, respectively, and by 78% in the neointima at day 7 after injury in ID- versus vehicle-treated group ( $P < 0.01$ ). ID significantly enhanced reendothelialization in the injured carotid arteries as determined by Evans blue stain and immunohistochemical analysis for von Willebrand factor. In the former assay, the reendothelialized area in injured vessels was 43% in ID-treated group versus 24% in the vehicle group ( $P < 0.05$ ); in the latter assay, the numbers of von Willebrand factor–positive cells per cross section increased from 24.8 (vehicle) to 60.5 (ID) ( $P < 0.01$ ) at day 14 after injury. In addition, the production of nitric oxide from excised carotid arteries was significantly higher in ID-treated than the vehicle group (8.5 versus 2.7 nmol/g,  $P < 0.01$ ). Finally, ID treatment reduced neointimal area and the ratio of intima to media by 45% and 40%, respectively ( $P < 0.01$ ), at day 14 after balloon angioplasty.

**Conclusions**—The results indicate that ID beneficially modulates the balloon denudation–induced vascular injury response. Inhibition of VSMC proliferation and acceleration of endothelial recovery likely mediate this protective effect of ID. (*Circulation*. 2000;102[suppl III]:III-281-III-288.)

**Key Words:** idoxifene ■ vasculature ■ endothelium ■ cells ■ restenosis

The vascular protective effect of estrogen was first shown in population studies in humans in which estrogen replacement therapy demonstrated a protective effect on atherosclerotic vascular diseases in postmenopausal women,<sup>1,2</sup> as later confirmed in ovariectomized (Ovx) monkeys. The vascular protective effect has since been documented in more detail in animal models and in vitro. The recent demonstration of estrogen receptor (ER) expression by vascular smooth muscle cells (VSMCs)<sup>3</sup> and

endothelial cells<sup>4</sup> has further suggested that estrogen may act directly on vascular tissue in addition to its cholesterol-lowering effect. Estrogen has been found to promote vasodilation both in humans and in experimental animals, accompanied by improved endothelial function.<sup>5</sup> Estrogen also exerts a direct inhibitory effect on smooth muscle by inhibiting calcium influx and reduces SMC proliferation that may attenuate the progression of atherosclerotic lesions.<sup>5</sup>

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Iodoxyfene (pyrrolidino-4-iodofamoxifene) is a novel selective estrogen receptor modulator (SERM) that is defined as a compound that has estrogen agonism on the desired target tissues such as bone and has antagonism or minimal agonism in reproductive tissue such as the breast or uterus. Iodoxyfene has a 2.4- to 5-fold-greater affinity for ER $\alpha$  than tamoxifen while being significantly less uterotrophic.<sup>6</sup> Iodoxyfene also binds to ER $\beta$  (unpublished data). A recent study<sup>7</sup> has demonstrated that idoxifene is an agonist through the estrogen response element and exhibits postreceptor effects similar to estrogen in bone-forming osteoblasts. Iodoxyfene can both prevent and arrest the loss of bone mineral density that occurs in the axial and appendicular skeleton in the Ovx rat. Iodoxyfene protected against Ovx-induced loss of bone mineral density in both the lumbar spine and the proximal tibia by suppressing the increase in bone turnover that occurs on estrogen withdrawal. Moreover, idoxifene showed an effect similar to estrogen by stimulating osteoclast apoptosis. These pleiotropic effects of idoxifene could contribute to the maintenance of bone homeostasis. However, idoxifene differs from estrogen in a tissue-specific manner. In human endometrial cells, in which estrogen is a potent agonist through the estrogen response element, idoxifene has negligible agonist activity and was able to block estrogen-induced gene expression in endometrial cells, which is in agreement with observations in the rat.<sup>7</sup> The pharmacological profile of idoxifene in animal studies suggests that it behaves like an ER agonist in bone and lipid metabolism while having negligible or no ER agonist activity on the uterus at doses that prevent bone loss. Therefore, idoxifene may provide a better profile for the treatment of postmenopausal diseases. However, it is unknown whether this SERM still retains the vascular protective effect of estrogen, an important therapeutic benefit for postmenopausal women who are known to be at increased risk for atherosclerotic cardiovascular diseases.

The present study was undertaken to explore the effect of idoxifene on balloon denudation-induced vascular injury response in Ovx rats in which idoxifene has been demonstrated to prevent bone loss while having negligible effect on the reproductive system. A well-established model of carotid artery balloon injury<sup>8</sup> was used because the cellular processes controlling the vascular response to injury are well characterized in this model and some of them may be also involved in atherosclerotic vascular diseases. The effects of idoxifene on injury-induced SMC proliferation, reendothelialization, and neointimal formation were studied at a dose that is effective for prevention of bone loss in the Ovx rat.<sup>7</sup> In addition, the effects of idoxifene on VSMC proliferation and endothelial cell apoptosis *in vitro* were also examined.

## Methods

### Cell Culture

Female human coronary SMCs (FHCSMCs) from an adult (age, 39 years) were purchased from Clonetics (lot 16810), and female rat aortic SMCs (FRASMCs) were isolated from medial explants from the thoracic aorta of female Sprague-Dawley rats as described previously.<sup>9</sup> The cells under passage 6 were used in this study. Bovine pulmonary arterial endothelial cells (BPAECs) (passages 17 through 20) were obtained from the American Type Culture Collection.

### DNA Synthesis

DNA synthesis in FHCSMCs was assessed by measurement of the incorporation of [<sup>3</sup>H]thymidine (New England Nuclear) as reported previously.<sup>9</sup> FHCSMCs were grown in 24-well plates in DMEM phenol-free media containing 10% charcoal-stripped FBS (Hyclone) and subsequently rendered quiescent with DMEM media without FBS for 48 hours. Iodoxyfene (SmithKline Beecham) 0.001 to 1  $\mu$ mol/L or vehicle was added 15 minutes before the FCSMCs were challenged with platelet-derived growth factor (PDGF; 1 nmol/L; Boehringer Mannheim) for 24 hours. [<sup>3</sup>H]thymidine (1  $\mu$ Ci per well) was added for a further 4-hour incubation. DNA synthesis was assessed by measuring the radioactivity incorporated into the trichloroacetic acid-insoluble fraction of the cells. The results were from 5 separate experiments performed in triplicate.

### Cell Proliferation Study

FHCSMCs were prepared as above and treated with vehicle or idoxifene (0.01 to 1  $\mu$ mol/L) for 15 minutes before the addition of PDGF (1 nmol/L), and incubation was continued for 5 days. The basal level of cell proliferation was measured from the cells treated with vehicle only. At the end of incubation, cells were harvested and counted.<sup>9</sup> The results were from 5 separate experiments performed in triplicate.

### Cell Cycle Study by Fluorescence-Activated Cell Sorting Analysis

FRASMCs were cultured in 6-well plates and made quiescent for 72 hours in phenol-free DMEM without FBS. Iodoxyfene (100 nmol/L) or vehicle was added to the cells 30 minutes before the addition of FBS (10% final) and incubated for 48 hours. At the end, cells were suspended in methanol ( $1 \times 10^6$  cells/mL) and stained with propidium iodide. The DNA content of the cells was analyzed by a Becton-Dickinson FACSCalibur flow cytometer. Fluorescent measurements collected from the cells were analyzed by the Cellquest (BDIS) data analysis software, followed by cell cycle analysis of DNA histograms with ModFitLT.<sup>10</sup>

### Morphological Assessment and Quantification of Apoptosis in Endothelial Cells

BPAECs were cultured in phenol-free DMEM containing 2% estrogen-free serum for 24 hours and treated with vehicle or idoxifene (100 nmol/L) for 30 minutes before the addition of tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ; 5 ng/mL) and incubation continued for the indicated times (time dependence), or the cells were treated with vehicle or idoxifene at the concentrations indicated in Figure 3B for 30 minutes before the addition of TNF- $\alpha$  (5 ng/mL) and incubation continued for 24 hours (concentration dependence). To quantify endothelial cells undergoing apoptosis, cell monolayers were fixed and stained with Hoechst 33324 (molecular probe). The morphological features of apoptosis (cell shrinkage, chromatin condensation, blebbing, and fragmentation) were monitored by fluorescence microscopy. At least 500 cells from 13 randomly selected fields per dish were counted as described previously.<sup>11</sup>

### Rat Carotid Artery Balloon Angioplasty

All procedures were reviewed and approved by the Animal Care and Use Committee at SmithKline Beecham Pharmaceuticals. Virgin female Sprague-Dawley rats (Charles River) at 7 to 9 months of age (weight, 350 to 400 g) were bilaterally ovariectomized 1 week before study<sup>7</sup> and then treated orally with either idoxifene (1 mg/kg, once a day) or vehicle for 3 days before balloon angioplasty. Iodoxyfene was suspended in 1% aqueous solution of carboxymethyl cellulose as described previously.<sup>7</sup> Left common carotid artery balloon angioplasty was performed under aseptic conditions,<sup>8</sup> and daily oral administration of idoxifene or vehicle was continued after surgery until the animal was killed. The animals were killed on days 1, 3, 7, and 14 after surgery for proliferating cell nuclear antigen (PCNA) study or at day 14 for reendothelialization or neointimal formation studies (see below).

### Neointimal Formation

The extent of neointimal formation was quantified histologically in left common carotid arteries 14 days after balloon angioplasty as described previously.<sup>8</sup> After the carotid arteries were removed from each rat,  $\approx 0.5$ -cm segments from both ends were removed before embedding in paraffin wax. This approach avoided removal of the areas close to edges where the damage could be low and give false-positive results. Four equidistant sections per artery were measured for entire circumference, lumen, media, and neointima with a Bioscan Optimas cell imaging system.<sup>8</sup>

### Evaluation of SMC Proliferative Activity in Injured Carotid Artery (PCNA Assay)

The injured arterial segments were harvested, and the SMC proliferative activity was evaluated by immunohistochemical analysis for PCNA as described previously.<sup>12,13</sup> The extent of proliferative SMCs was determined by counting PCNA-positive SMCs in intima and media. Four sections from each artery and 10 fields per section were reviewed and scored by a board-certified pathologist. The percentage of PCNA-positive cells versus total counted cells was calculated.

### Evaluation of Reendothelialization by Evans Blue Staining

Two weeks after angioplasty, the Ovx rats treated with idoxifene ( $1 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{d}^{-1}$ ) or vehicle were anesthetized and injected with sterile Evans blue dye via the femoral vein. A half-hour later, the animal was perfused with saline in situ at a pressure of 90 mm Hg until the effluent ran clear, followed by 5 minutes of fixation with 100% methanol. The carotid arteries were harvested as described above, incised longitudinally, and then pinned to a tray containing wax for photography with a dissecting microscope. Planimetric analysis with an Optimas 6.2 Image analysis program was performed. The reendothelialized area was defined macroscopically as the area not stained with Evans blue dye. Reendothelialization was expressed as reendothelialized area versus the total denuded area as described previously.<sup>13</sup>

### Detection of Reendothelialization by von Willebrand Factor Immunohistochemistry

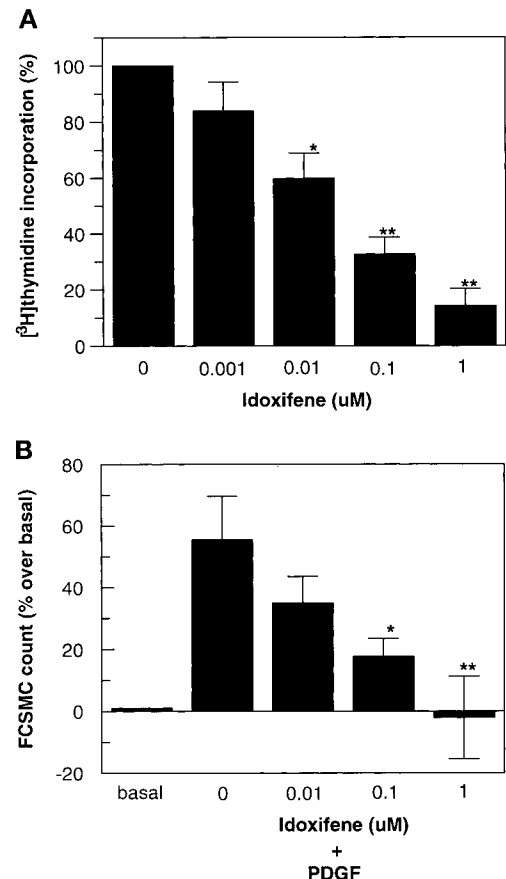
The carotid arteries were harvested and processed as described above. Tissue ( $5\text{-}\mu\text{m}$ ) tissue slides were deparaffinized, rehydrated, and placed in PBS with 0.05% Tween. The primary antibody used was a rabbit anti-von Willebrand factor (DAKO) followed by an anti-rabbit IgG biotinylated secondary (Vector Laboratories). Tissues were incubated in a dilution of streptavidin-hrp (DAKO) followed by 3'3'-diaminobenzidine (DAB). The slides were counterstained with hematoxylin, dehydrated, and placed in coverslips. Six fields per section and 4 sections per artery were counted (6 rats per group).

### Measurement of Nitric Oxide From Vessel Segments

To determine whether functional recovery of the endothelium was accelerated by idoxifene, the production of nitric oxide by excised carotid arterial segments was measured by modification of a method reported previously.<sup>14</sup> The balloon-injured carotid arteries were collected from the Ovx rats at day 14 after injury. Vascular segments were rinsed with PBS, cleaned of connective tissue, and homogenized in PBS (1:100, wt:vol). The nitric oxide concentrations in the supernatant were quantified by a chemiluminescence detector (Sievers 270B NO Analyzer).

### Data Analysis

Statistical analysis was performed by Dunnett's test with a program provided by MicroComputer Specialists. A value of  $P < 0.05$  was considered significant.



**Figure 1.** Inhibition by idoxifene of PDGF-induced DNA synthesis ( $[\text{H}]$ thymidine incorporation) (A) and proliferation (B) in FHC-SMCs. FHC-SMCs were rendered quiescent in serum- and phenol-free DMEM for 48 hours. Idoxifene or vehicle was added 15 minutes before addition of PDGF ( $1 \text{ nmol/L}$ ). A, Incubation continued for 24 hours, and  $[\text{H}]$ thymidine ( $1 \mu\text{Ci}$  per well) was added for 4 more hours. DNA synthesis was assessed by measuring radioactivity incorporated into trichloroacetic acid-insoluble fraction of cells. B, Incubation continued for 5 days, and cell proliferation was expressed as percent change of cell count over nonstimulated cell count (basal). Results are from 5 separate experiments performed in triplicate. \* $P < 0.05$ , \*\* $P < 0.01$  vs PDGF-treated sample alone.

## Results

### Inhibition of PDGF-Stimulated DNA Synthesis and Proliferation in FCSMCs

In FCSMCs, PDGF stimulated a significant increase in  $[\text{H}]$ thymidine incorporation into the cells that was similar to that reported previously.<sup>9</sup> The count-per-minute values in vehicle and PDGF-treated FCSMCs were  $719 \pm 78$  and  $1616 \pm 162$  ( $P < 0.01$ ,  $n = 5$ ), respectively. Idoxifene exerted a concentration-dependent inhibition of PDGF-stimulated  $[\text{H}]$ thymidine incorporation with an  $\text{IC}_{50}$  value of  $20.4 \text{ nmol/L}$  (Figure 1A). As shown in Figure 1B, idoxifene inhibited PDGF-induced FCSMC proliferation ( $\text{IC}_{50} = 27.5 \text{ nmol/L}$ ).

### Inhibition of Serum-Induced Cell Cycle Progression in FRASMCs

Serum-stimulated cell cycle progression in quiescent FRASMCs and the effect of idoxifene are shown in the Table

**Idoxifene Arrests Female Rat Aortic Smooth Muscle Cell Cycle at S Phase**

	Cell Cycle Stage		
	G <sub>0</sub> /G <sub>1</sub>	S	G <sub>2</sub> /M
Basal	95.0±0.9	4.4±0.8	0.6±0.1
Serum	71.2±4.5*	16.0±6.8*	12.8±7.4‡
Serum + idoxifene (100 nmol/L)	68.2±2.5‡	31.6±1.5‡	0.2±0.2§

n=3.

\**P*<0.05; †*P*<0.01, ‡*P*<0.01 vs basal; §*P*<0.01 vs serum.

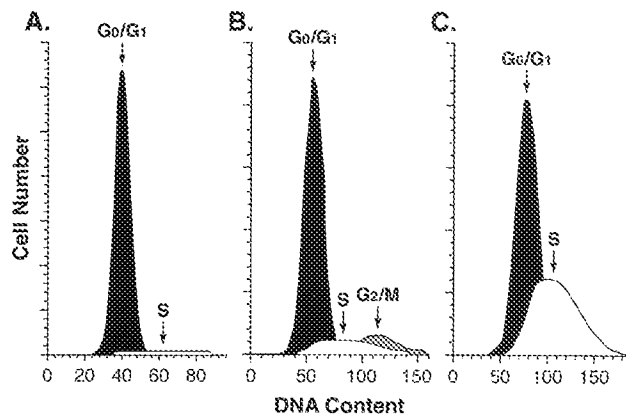
(n=3). In the presence of 100 nmol/L idoxifene, the entering of SMCs into G<sub>2</sub>/M from S phase was completely abrogated with a concomitant increase in the percentage of cells in S phase, indicating that idoxifene arrested the cells in S phase. Figure 2 is a representative measurement of DNA distribution in serum-stimulated FRASMCs in the absence or presence of 100 nmol/L idoxifene.

**Idoxifene Protects Endothelial Cells From TNF- $\alpha$ -Induced Apoptosis In Vitro**

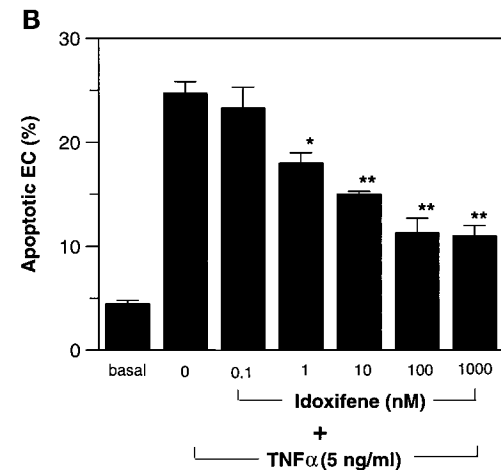
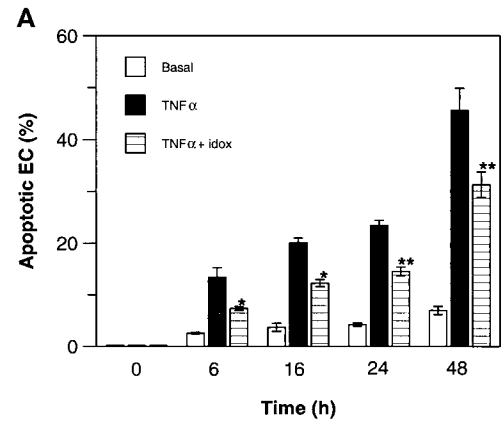
Endothelial cells exposed to TNF- $\alpha$  (5 ng/mL; Genzyme) and stained with Hoechst 33324 displayed morphological features of apoptotic cell death: condensed chromatin, fragmented nuclei, and blebbing of the plasma membrane as reported previously<sup>11</sup> (data not shown). Figure 3A demonstrates a time dependence of TNF- $\alpha$ -induced apoptosis in endothelial cells and the protective effect of idoxifene at 100 nmol/L. Figure 3B shows a concentration-dependent protection by idoxifene of TNF- $\alpha$ -induced endothelial cell apoptosis. The number of apoptotic cells over the basal level was reduced by 66.3% in the presence of 100 nmol/L idoxifene.

**Inhibition of Transluminal Balloon Injury-Induced Carotid Artery SMC Proliferation**

Proliferative SMCs in the injured carotid arterial segments harvested on days 1, 3, 7, and 14 after injury were evaluated

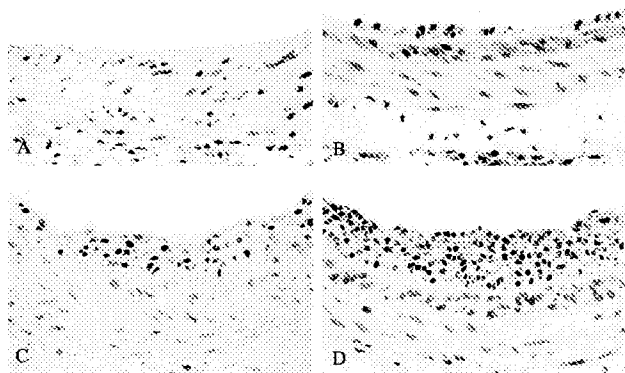
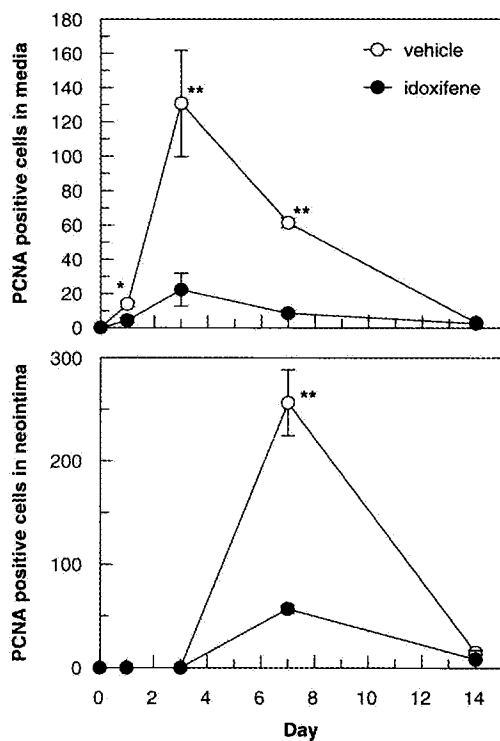


**Figure 2.** Representative measurement of DNA distribution in serum-stimulated FRASMCs in absence or presence of idoxifene (A; unstimulated cells). FRASMCs were quiescent for 72 hours. Vehicle (B) or idoxifene (100 nmol/L; C) was added 30 minutes before addition of FBS (10% final), and incubation continued for 48 hours. Cells were harvested and stained with propidium iodide. DNA content of cells was analyzed with Becton-Dickinson FACSCalibur flow cytometer.



**Figure 3.** Protection by idoxifene of TNF- $\alpha$ -induced apoptosis in endothelial cells (EC). BPAECs were cultured in phenol-free DMEM containing 2% estrogen-free serum for 24 hours and treated with vehicle or idoxifene (idox; 100 nmol/L) for 30 minutes before addition of TNF- $\alpha$  (5 ng/mL) and incubation continued for indicated times (A), or cells were treated with vehicle (basal) or idoxifene at indicated concentrations for 30 minutes before addition of TNF- $\alpha$  (5 ng/mL) and incubation continued for 24 hours (B). Cells were fixed and stained with Hoechst 33324. Quantification of apoptotic BPAECs was based on morphological features of apoptosis as described previously.<sup>11</sup> \**P*<0.05, \*\**P*<0.01 vs vehicle treated (n=5).

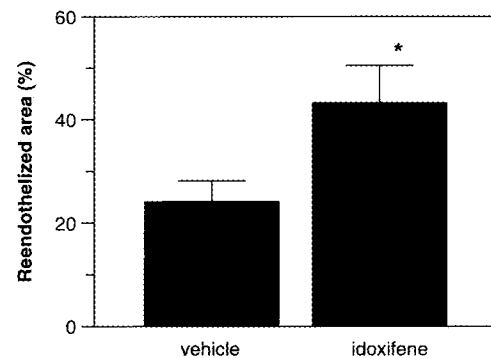
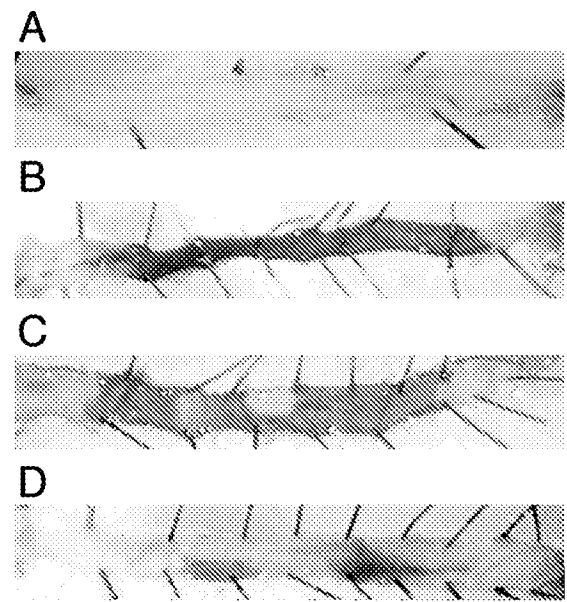
by histochemical analysis for PCNA. In vehicle-treated animals, the number of PCNA-positive SMCs in the media was significantly increased on days 1, 3 (peak), and 7 after injury and then reduced to an undetectable level (Figure 4A). In the idoxifene-treated group, an increased number of PCNA-positive SMCs was observed on day 3 after injury but to a much smaller degree compared with idoxifene-treated rats (Figure 4A). A remarkable increase in the number of PCNA-positive SMCs was also demonstrated in the intima 7 days after injury in vehicle-treated rats, which was much higher than the number of PCNA-positive SMCs in idoxifene-treated group at the same time (Figure 4B). Figure 4C is a representative photomicrograph demonstrating PCNA immunostaining in the intima and media on days 3 and 7 after balloon injury in idoxifene- (A and B) and vehicle- (C and D) treated rats.



**Figure 4.** Inhibition of transluminal balloon injury–induced carotid artery SMC proliferation in Ovx rats. Idoxifene ( $1 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{d}^{-1}$ ) was administered orally 2 days before angioplasty and continued up to 2 weeks. Injured carotid arteries were harvested at days 1, 3, 7, and 14 after angioplasty, and proliferative SMCs in media (A) or neointima (B) were detected by PCNA method. Extent of proliferative SMCs was determined by counting PCNA-positive cells in intima and media (10 fields per section), and percent of PCNA-positive cells versus total counted cells was calculated.  $*P < 0.05$ ,  $**P < 0.01$  vs idoxifene-treated group. C, Representative photomicrographs of cross sections of rat left carotid arteries at days 3 (A, C) and 7 (B, D) after angioplasty in idoxifene- (A, B) and vehicle- (C, D) treated rats. Brown stain cells are PCNA positive.

#### Idoxifene Enhances Reendothelialization Detected by Evans Blue Stain and Von Willebrand Factor Immunohistochemical Analysis

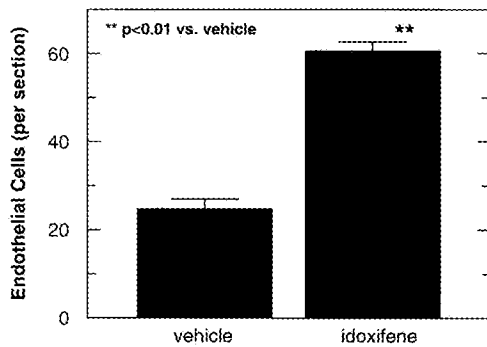
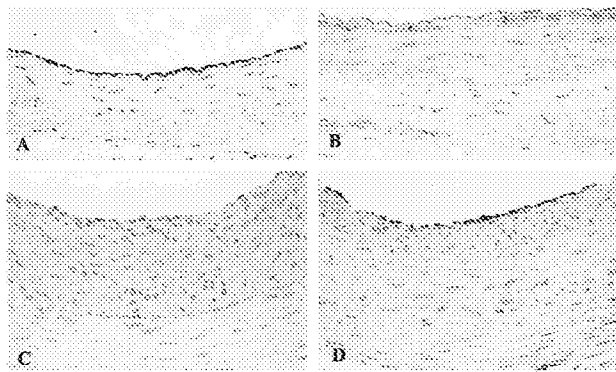
Figure 5 (top) illustrates the examples of left carotid arteries harvested from sham-operated or balloon angioplasty rats treated with vehicle or idoxifene. Evans blue staining identifies segments of each injured carotid artery that has not been reendothelialized. No staining was found in sham-operated



**Figure 5.** Extent of reendothelialization in injured carotid arteries by Evans blue staining. Top, Representative arteries harvested immediately (B) or 2 weeks after denudation in vehicle-treated (C) or idoxifene-treated (D) rats. A, Uninjured vessel from sham-operated rat. Evans blue staining identifies segments of each artery that have not been recovered by endothelium. Total reendothelialized area was measured and expressed as percentage of total damage area. Bottom, Quantitative result from vehicle- and idoxifene-treated groups.  $*P < 0.05$  vs vehicle ( $n = 12$ ).

vessel (A), but almost the entire area of the artery harvested immediately after denudation was stained by Evans blue (B). At 2 weeks after denudation, the area of reendothelialization in vehicle-treated artery (C) was significantly smaller than that in idoxifene-treated artery (D). The bottom panel of Figure 5 shows the quantitative results. The percentage of area in injured carotid arteries covered by endothelium was nearly 2-fold higher in the idoxifene-treated group than that observed in the vehicle-treated group ( $P < 0.05$ ,  $n = 12$ ).

Figure 6 shows the results of immunohistochemical study for von Willebrand factor (factor VIII). Sections of uninjured left carotid arteries showed a linear immunofluorescence staining for factor VIII in the endothelium but not in the SMCs or fibroblasts of the media, as shown in the top panel (A). The linear staining for factor VIII was not observed in the cross section of the left carotid artery 1 day after denudation (B). The cross section of the injured carotid artery



**Figure 6.** Determination of reendothelialization in injured carotid artery by factor VIII immunohistochemistry. Top, Representative photomicrographs from arteries harvested from sham (A), immediately (B) or 2 weeks after denudation in vehicle- (C) or idoxifene- (D) treated rats. Bottom, Quantitative result from vehicle- and idoxifene-treated groups ( $n=6$ , 4 sections per artery). \*\* $P<0.01$  vs vehicle.

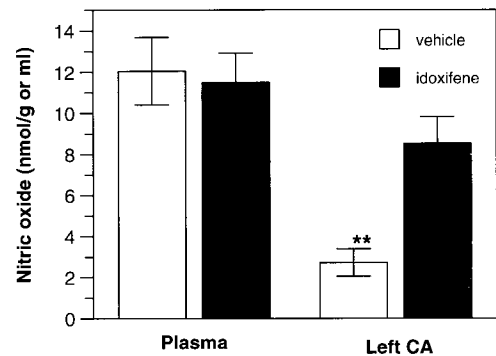
from the idoxifene-treated rats (D) but not from the vehicle-treated rat (C), showed a linear positive staining for factor VIII on the surface of the intima. The bottom panel is the quantitative measurement of the number (per section) of endothelial cells (factor VIII-positive cells) in the left carotid arteries from idoxifene- and vehicle-treated rats (6 fields per section, 4 sections per rat). The number of endothelial cells per section was increased from  $24.8 \pm 2.2$  (vehicle) to  $60.5 \pm 2.1$  (idoxifene) ( $P<0.01$ ,  $n=6$ ).

#### Nitric Oxide Production Is Increased in Idoxifene-Treated Arteries

Nitric oxide production by the injured left carotid arteries was significantly increased from  $2.71 \pm 0.67$  (vehicle) to  $8.52 \pm 1.31$  (idoxifene) nmol/g tissue ( $P<0.01$ ,  $n=12$  to 13) at 14 days after balloon angioplasty as shown in Figure 7. There was no difference in the plasma levels of nitric oxide between the 2 groups.

#### Idoxifene Reduces Neointimal Formation After Carotid Denudation in the Ovx Rat

The total neointimal area of the left carotid arteries measured 2 weeks after balloon denudation was  $0.13 \pm 0.01$  mm<sup>2</sup> in vehicle-treated animals compared with  $0.07 \pm 0.01$  mm<sup>2</sup> in the idoxifene-treated group (45% reduction,  $P<0.01$ ,  $n=15$ ). The calculated intima/media ratios also displayed a significant decrease in the idoxifene-treated animals compared with the



**Figure 7.** Idoxifene enhances nitric oxide formation in balloon-injured carotid artery (CA) in Ovx rats. Left carotid arteries and plasma were harvested 2 weeks after injury from vehicle ( $n=12$ ) and idoxifene-treated ( $n=13$ ) Ovx rats, and nitric oxide was measured. \* $P<0.01$  vs idoxifene-treated group.

vehicle group ( $P<0.01$ ) (Figure 8, top). The bottom panel of Figure 8 shows representative cross sections of left carotid arteries from a sham-operated rat (A), a vehicle-treated rat (B), and an idoxifene-treated rat (C) 14 days after angioplasty.

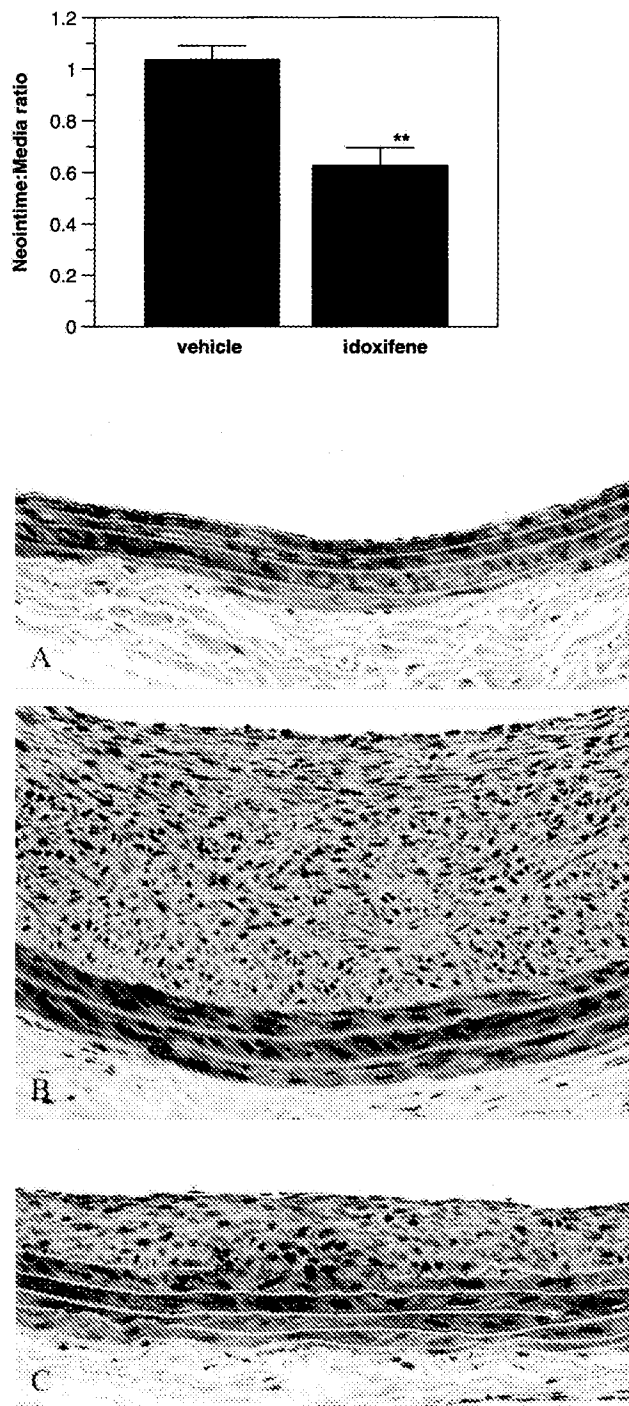
#### Discussion

The concept of SERM has been demonstrated for a number of compounds, including idoxifene. However, the molecular mechanisms for the tissue-specific effect of SERM are not well understood. Two types of ER (ER $\alpha$  and ER $\beta$ ) have been identified, and their tissue distribution and functions have been the focus of intense studies.<sup>15,16</sup> Adding to the complexity, recent studies have indicated that transcriptional regulation by ER depends on a variety of coactivators and corepressors, and more novel proteins may be involved.<sup>17,18</sup> The effect of SERM on these cofactors is not known. Because of this complexity, the profile of tissue-specific effects of each SERM is still unpredictable and was based mainly on animal and clinical studies. The objective of the present study is to find whether idoxifene, a new SERM, retains vascular protective effects of estradiol.

VSMC proliferation is one of the major vascular remodeling processes after vascular injury that may participate in the vaso-occlusive disorders associated with multiple vascular diseases.<sup>19</sup> In the present study, the antiproliferative effects of idoxifene on VSMCs were demonstrated in vitro and in vivo. Medial SMC proliferation appeared immediately after balloon injury and peaked at day 3 (Figure 4), which is consistent with the observation reported previously.<sup>20</sup> Proliferative VSMCs also appeared in the intima 5 to 7 days after injury when the intimal formation was clearly demonstrated. The selective ER modulator idoxifene significantly reduced the number of proliferative VSMCs in both the media and intima. In vitro studies further demonstrated a direct inhibitory effects of idoxifene on PDGF-stimulated VSMC proliferation, similar to that reported in 17 $\beta$ -estradiol-treated VSMCs.<sup>21</sup>

We were interested in exploring further the hypothesis that ER may exert an effect on cell cycle progression in VSMCs. The data presented in the Table and Figure 2 indicate clearly that cells treated with idoxifene encountered an S-phase arrest accompanied by a gradual decrease in SMCs from the G<sub>2</sub>/M





**Figure 8.** Effect of idoxifene on neointimal formation after balloon injury in carotid artery of Ovx rats. Idoxifene ( $1 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{d}^{-1}$ ) or vehicle was administered daily by gavage. Animals were killed 14 days after angioplasty. Carotid artery intimal and media areas were determined, and ratios of intima versus media from each animal were calculated (top). Data represent mean  $\pm$  SEM ( $n=15$ ). Bottom, Representative cross sections of left carotid arteries from sham (A), vehicle-treated (B), and idoxifene-treated (C) Ovx rats 14 days after angioplasty. \*\* $P<0.01$  vs vehicle.

phase. The data agree with the results obtained from in vitro [ $^3\text{H}$ ]thymidine incorporation and proliferation studies. The cell cycle progression of SMCs from S to G<sub>2</sub>/M phase was hindered by idoxifene leading to an overall reduction in the total number of cells undergoing cell division. As a result,

[ $^3\text{H}$ ]thymidine incorporation was decreased significantly in idoxifene-treated SMCs.

Endothelial cells affect the homeostasis of the vessel wall in terms of vasomotor tone, platelet and monocyte adhesion, growth of SMCs, and extracellular matrix production and thereby provide an antithrombotic and anti-inflammatory barrier for the normal vessel wall. Disruption of the anatomic and functional integrity of the endothelium has been postulated as a mechanism for the initiation of atherosclerosis.<sup>19</sup> Recent data have suggested that after arterial injury, acceleration of reendothelialization by  $17\beta$ -estradiol is associated with attenuation of intimal hyperplasia.<sup>22,23</sup> Accordingly, we performed a series of experiments to determine whether idoxifene accelerates reendothelialization after arterial injury. Two weeks after balloon injury, the idoxifene-treated animals showed more rapid recovery of the endothelium and its barrier function as demonstrated by exclusion of Evans blue dye and immunohistochemical study for factor VIII in the vessel. Furthermore, recovery of endothelial function, manifested as enhanced nitric oxide production, was also shown to be accelerated by idoxifene treatment. The data are consistent with recent studies that demonstrated that  $17\beta$ -estradiol accelerated reendothelialization after arterial injury.<sup>22,23</sup>

It has been suggested that increased endothelial cell turnover mediated through accelerated apoptosis plays a role in endothelial disruption.<sup>24</sup> To further explore the mechanisms for the enhancement of reendothelialization by idoxifene, we studied the effect of idoxifene on TNF- $\alpha$ -induced apoptosis in endothelial cells. TNF- $\alpha$  is known to be secreted by macrophages, activated T cells,<sup>25</sup> and SMCs after vascular injury.<sup>26</sup> TNF- $\alpha$ , as a proapoptotic factor for endothelial cells, has been demonstrated in vitro in human and nonhuman species.<sup>24,27</sup> The reported upregulation of TNF- $\alpha$  expression in animal models of arterial injury by balloon angioplasty<sup>26</sup> and in human coronary artery restenotic lesions<sup>28</sup> provides further evidence that regulation of the expression of this cytokine may be functionally important in vivo.<sup>29</sup> The data presented in Figure 3 demonstrate the protection by idoxifene of endothelial cell apoptosis induced by TNF- $\alpha$ . The protective effect was even observed at  $1 \text{ nmol/L}$  idoxifene. However, the maximal protection by idoxifene was 66%, and increasing the concentration of idoxifene did not further enhance the protective effect, indicating that not all proapoptotic signals implicated in TNF- $\alpha$ -induced apoptosis were affected by idoxifene. Our data are in accordance with a recent study that demonstrated the protective effect of  $17\beta$ -estradiol against TNF- $\alpha$ -induced apoptosis in cultured endothelial cells.<sup>29</sup> It has been suggested that estradiol, by protection of endothelial cell from apoptosis and enhancement of endothelial cell adhesion, may help migrating cells to colonize in injured vessel and aid in reendothelialization.<sup>24</sup> This could also be a mechanism for idoxifene in enhancing reendothelialization.

The enhanced regeneration of endothelial cells by idoxifene appeared to contribute to suppression of intimal hyperplasia. Animals in the idoxifene-treated group showed less intimal thickening than those in the vehicle-treated group, consistent with the reported data of  $17\beta$ -estradiol in the same model.<sup>30,31</sup> The notion that this observed differential in

neointimal thickening truly represents a differential degree of intimal hyperplasia is supported by the results of PCNA immunostaining. The number of PCNA-positive SMCs in both media and neointima was significantly higher in the vehicle-treated than idoxifene-treated group. The inverse relationship between reendothelialization and neointimal SMC proliferation indicates that neointimal thickening thus developed in association with delayed reendothelialization, and the vascular protection provided by idoxifene is most likely through inhibition of VSMC proliferation and acceleration of reendothelialization.

In summary, the present study has demonstrated for the first time the beneficial vascular remodeling effect of idoxifene in a balloon denudation rat model. Both inhibition of SMC proliferation and acceleration of endothelial regeneration likely mediate this vascular protection. The favorable effect of idoxifene on vessel wall injury, together with its cholesterol-lowering action,<sup>7</sup> would be expected to have implications for clinical benefit. The data from the present study also suggest that SERM is able to retain the favorable vascular protective effect while avoiding unwanted effects of estrogen on the reproductive system. This could be another therapeutic advantage to the use of SERM in postmenopausal women.

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